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CONTRACTING ORGANIZATION: University of California
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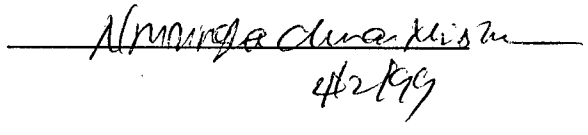

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13. ABSTRACT *(Maximum 200*

The purpose of this project was to develop a reliable, non invasive technique to gain access to the lining of the milk ducts. In a previous IDEA grant we demonstrated the feasibility of an intraductal approach to breast disease, the ability to obtain ductal cells through washings, and the general anatomy of the nipple duct orifices and ductal systems. In our first contract year, we demonstrated the ability to retrieve cells from women's ducts (pre and post mastectomy) through a newly developed double lumen catheter. After studying the microanatomy of the ductal orifices, we also began work on developing a technique for identifying the breast duct orifices. In this second contract year, we determined that our approach involving the dekeratinization of the nipple and application of a fluorescent keratin antibody to the ductal epithelium was not effective. A second approach was developed based on the random diffusion of a small molecular dye into the lactiferous sinuses of the ductal lumens. This method allows for the constant and reproducible identification of each and every duct. In addition, we have continued to refine the retrieval of duct cells in attached and detached breasts with refinements of the catheter as well as our techniques.

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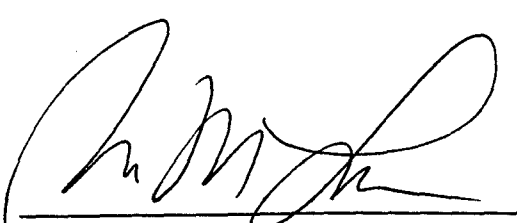

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INTRODUCTION

Subject: The subject of this work is a new approach to the study, detection and treatment of breast cancer.

Purpose: The purpose of this project is to develop a reliable non-invasive technique to gain access to the lining of the milk ducts.

Scope:
Project one: anatomy

1. devising a local approach to highlighting the ducts *(completed)*
   a. microanatomy of the nipple
   b. dekeratinization/fluorescence (non-viable approach)
   c. random diffusion/capillary action (viable approach)
2. confirming the anatomy *(pending)*
   a. in cadavers
      1) casting
   b. digitally on MRI
   c. of the nipple duct orifices in the normal woman study
   d. of the ductal systems in the calcification and mastectomy studies

Project two: cell retrieval

1. use a prototype catheter in a woman about to undergo a mastectomy and demonstrate that we could get cells back *(completed)*
2. determine the optimal way to both retrieve cells and outline duct *(in process)*
3. demonstrate that the whole duct was being washed
4. devise a technique to discriminate premalignant calcifications by washings *(pending)*

Background: One limiting factor for all of breast cancer research and treatment has been our lack of knowledge about the anatomy of the breast and our lack of intermediate markers of breast cancer risk. The main reason we have been unable to identify such markers is that we do not have ready, reproducible non-invasive access to the lining epithelium where breast cancer starts. Breast duct endoscopy and pathological/ cytological analysis has the potential to be a means of gaining access to the ductal epithelial cells and diagnosing, treating and studying the precancerous state as well as demonstrating the normal anatomy.

The purpose of our previous IDEA grant (DAMD1794-J-4281)was to demonstrate the feasibility of this approach. We progressed through three stages:

1) Demonstrating the feasibility of intraductal endoscopy with a flexible ductoscope;
2) Demonstrating the ability to retrieve ductal epithelial cells;
3) Describing the normal anatomy of the breast ducts and the nipple orifices.

Initially we attempted breast duct endoscopy in nine patients and were able to cannulate seven. We had difficulty obtaining washings through aspiration from the duct but were able to retrieve fluid from the surface of the nipple through a capillary tube. In five of the nine cases we obtained epithelial cells in the washings. In one the cells were consistent with proliferative disease. In three there was atypical epithelium and in one there were frank ductal carcinoma cells. There were no complications or untoward events in these first nine patients. In addition to the technical observations we also demonstrated a number of important observations with respect to both the normal ductal system as well as ductal carcinoma *in situ* (DCIS). In the studies where contrast dye was injected, the ductal system appeared to be a non-anastomosing three dimensional network composed of ducts and acini which are difficult to correctly assign to their ductal system of origin in routine two dimensional histological sections. In studies where several different dyes were injected in different ductal systems, DCIS appeared confined to a single ductal system despite the illusion of being present within a number of different ducts on routine sections.

In phase two of the IDEA grant we found that we could identify one half to one third of the ductal orifices (assuming 5-9) and obtain ductal cells from one third of the ducts washed. In three cases we were able to analyze cells for DNA, ploidy, G actin and EGF. We were unsuccessful obtaining cells in washings in 19 patients (51%). Because of either technical difficulties (not being able to dilate the duct sufficiently, not being able to instill saline, not being able to withdraw saline) or acellular return.

In our anatomical studies we found that the median number of lactating duct orifices identified per breast was 5 with a range of 1-12. This corresponds with earlier estimates by Sartorius of 6-12. The pattern of duct orifices and ductal systems is best displayed as a three dimensional cone with ducts extending not radially but toward the chest wall in two concentric circles. The inner group of four ductal systems is fairly constant as is one extending into the upper outer quadrant. The variability comes with the peripheral systems in the upper lower and medial breast.

From this initial work we concluded that:

- Breast duct endoscopy is feasible and cells can be obtained from the ducts for analysis. Although technical difficulties preclude this from being a clinically useful technique at this time, it has yielded important pathological and anatomic observations that are clinically relevant.
- The median number of lactating breast duct orifices per nipple is 5 with a range from 1-12. They form a consistent pattern that is bilateral. The breast ducts form a non-anastomosing three dimensional network composed of ducts and acini which do not correspond to quadrants or radial wedges and are difficult to correctly assign to their duct of origin in routine two dimensional histological sections. Rather they project back towards the chest wall in a pattern of two concentric circles.
- Although DCIS appears to be confined to one ductal system, the anatomic complexity bears directly on our ability to gauge the area that needs to be removed as well as the accuracy of both positive and negative margins. Further confirmation of this anatomical pattern will be important in designing breast surgery for DCIS.

The current contract is an extension of this work. In the first year we demonstrated the utility of the double lumen catheter in retrieving cells from the milk ducts in breasts that had been surgically removed and in women undergoing biopsies. We also studied the microanatomy of the nipple and devised a possible approach to identify the milk duct orifices using a fluorescent antibody. This year we continued with both of these projects with increasing success.

BODY

Project one:

1. Lighting up the nipple duct orifices

Background: Although our previous analysis of the anatomical patterns and general number of ducts was useful information, it did not help us in an individual case. Knowing that most women have 13 or less ductal orifices doesn't help us when confronted with an individual woman. We still needed a technique which would mark all of the nipple duct orifices in any one woman. This year we put most of our efforts into this problem. We explored several approaches which were not successful before coming up with a viable approach. Each approach yielded some interesting observations. They included:

a) Dekeratinization and application of fluorescent antibodies
b) Natural fluorescence
c) Nitrocellulose filters
d) Transareolar dye injection

a) Dekeratinization and application of fluorescent antibodies (non-viable)

Our ability to selectively stain the ductal glandular epithelium led us to hypothesize that we could selectively stain the ductal epithelium and then treat it with a fluorescent antibody which would be visible at the nipple surface. This would achieve our task of identifying the nipple duct orifices. In the first year we developed this technique in a step wise fashion employing pig nipples (a readily available hairless animal model). We developed a dekeratinization technique using acetic acid which removed the keratin layer without damage to the epithelial tissue. We then attempted to develop a technique for specifically fluorescing the ducts using a primary antibody of mouse anti-human epithelial membrane antigen (EMA) and a secondary antibody of goat anti mouse IgG (H and L chains) FITC conjugate. To visualize the FITC probe, we used a xenon lamp (150W) with a blue light filter (488nm), argon laser (protective) goggles (514nm). This fluorescent procedure only resulted in diffuse fluorescence to date which was demonstrated to be due to diffuse nonspecific binding. (See Table 1, Appendix.)

b) Natural Fluorescence (non-viable)

While looking for fluorescence caused by the antibodies noted above, we observed a natural fluorescence of the nipple. We were able to demonstrate that nipple aspirate fluid also exhibited fluorescence and explored whether this natural fluorescence could be used to identify ducal orifices. Multiple observations of women and men

demonstrated that the fluorescence was secondary to keratin and not specific for the breast duct orifices.

c) Nitrocellulose filters (non-viable)

Imayama, et al[1] reported the application of nitrocellulose filters for 24 hours to the nipples of women who had had breast cancer. Subsequent analysis of the filter paper demonstrated increased levels of CEA. This suggested that there is an imperceptible leakage of fluid from the nipple over time. We collected nitrocellulose filters from a number of volunteers with the hope that we could capitalize on this leakage to develop a map of the orifices on the filter paper. Although we were able to confirm Inaji (?)'s finding of evidence of nipple aspirate fluid there was also transfer of keratin to the paper. We were not able to identify a consistent marker which would make this a viable approach.

d) Transareolar dye injection (viable)

This method is based on several observations that were made while attempting to identify and map the duct orifice. The key observations were procured during practice and histologic analysis of specimens used in the earlier approaches.

The ducts are designed to secrete and deliver products to the surface of the nipple. The natural design of the mammary ductal system hinders the opposing direction with a a keratin plug that occludes the ductal orifice(s) and protruding surface epithelium that acts as an incomplete valve. At the base of the nipple there are lactiferous sinuses that taper as they course to the surface of the nipple. This approach uses the natural wicking of the lactiferous sinuses toward the surface of the nipple to identify the ductal orifices.

Method:

a.) 1-2 cc's of lymphazurin, a small molecular dye, is introduced transareolarly into the base of the nipple with a syringe. By random diffusion the dye enters the ductal lumens of the lactiferous sinuses.
b.) The tapering nature of the ductal lumens as they course to the surface wicks the dye to the surface of the nipple via capillary action.
c.) The ductal orifice(s) are identified at the surface of the nipple as deep blue pinpoints within 10-20 seconds with all appearing within 2-3 minutes.
d.) The potential ductal orifices are then cannulated with a guidewire or cannula and then a single or double lumen catheter
e.) Tissue specific marking dyes are then instilled into the ducts
f.) The breast is processed for histology and serial horizontal sections under the nipple are taken to demonstrate the duct profiles. Ducts successfully identified and cannulated would display ink within the ductal lumen.

Results:

This method was attempted in a series of 13 detached breasts (fresh breasts which had just been surgically removed) (see Table 2, Appendix). Allowing for a learning

curve we were able to identify all of the ducts and confirm them allows for the constant and reproducible identification of each and every duct. Water-resistant dye of both the same color and different colors support several conclusions about ductal anatomy-nipple anatomy:

1) there are between 5 and 9 ducts/nipple
2) the ductal systems are separate and non-anastomosing
3) serial sections of the nipple confirmed that our technique identifies all ductal orifices
4) histology demonstrated that the dye can reach the far recesses of the ductal lobular unit

The procedure was repeated in a woman using lymphazurin mixed with lidocaine. The procedure was well tolerated and eight ductal orifices were identified and cannulated without difficulty.

Project 1: 2. Confirming the anatomy

a) cadaveric studies: this project was put on hold until we were able to reliably identify all of the ductal orifices. Now that this technique has been developed, we are proceeding with developing a three dimensional cast of the breast.

b) MRI: using the dye injection technique in a volunteer, MRI ductograms were done on four ducts in one breast. Gadolinium was used for three of the studies and proved to diffuse out of the ductal system very rapidly. The fourth study was done with conray and was more successful. This study demonstrated several findings:
   - The duct orifices correlated to the ductal systems in the same area of the breast; i.e., the orifice at twelve o'clock corresponded to the ductal system that was located in the upper portion of the breast.
   - There were no inter-duct connections
   - Gadolinium diffused out of the ducts quickly

   We plan to repeat this study using conray.

c) Correlation of ductal anatomy with our previous lactation/ductogram studies

We are in process of collecting all of the data collected in our human studies for statistical analysis which will be completed in year three.

Project 2: cannulation of the duct orifices

Background: In our IDEA grant we devised a double lumen catheter which would allow a continuous flow of saline through out the ductal system. The prototype catheter was a 3 French double lumen catheter. The proximal lumen is smaller in diameter and is used for instilling saline; the distal lumen is larger and is used for aspiration. We initially studied detached breasts, or breasts which had just been removed surgically but not yet sent to pathology. We assumed that these fresh breasts would still have intraductal cells which could be retrieved through washings. We had determined that cells could be retrieved and preserved in 80% of the 10 breasts we had studied. In addition we studied six attached breasts (prior to mastectomy or breast surgery).

Materials and methods:
Mild suction was applied to the nipple to try and elicit discharge. A dissecting microscope or loupes were used to magnify the nipple. A map was made of the identified orifices. Starting with the most promising orifice (i.e., most amount of discharge, largest) we attempted to cannulate it using either a standard set of metal dilators (galactography set by Mahan), a very small glide wire (type used in angiography). Once the duct had been cannulated and dilated to approximately 0.7-1.0 cm, the double lumen catheter was threaded into the duct. Saline was instilled setting up a continuous flow until 10cc have been collected. This procedure takes approximately 15 minutes. If we were unable to complete the procedure within the 15-minute limit, we stopped prematurely. The washings were then sent to cytology for analysis.

This year we extended these studies (see Table 3). We have observed:

- Better yield in the attached breast than the detached one
- Better yield with the double lumen catheter than with single lumen catheters used in previous studies (1000-2000 series).

This has allowed us to refine the catheter and the evaluation of cytology. We have now applied to use our duct identification technique in these studies to improve our yield.

CONCLUSIONS

This past year has seen two key accomplishments:

1. Development of a method for the constant and reproducible identification of each and every ductal orifice. This method has confirmed the following:
    - there are between 5 and 9 ducts/nipple
    - the ducts are separate and non-anastomosing
    - serial sections of the nipple confirms that our technique identifies all ductal orifices
    - additional studies confirm that the dye can reach the far recesses of the ductal lobular unit
2. Development of a reliable method for cell retrieval.

The final year of the contract will involve completing the scope of work:

1. Confirm anatomical studies
    a) cadaver three dimensional cast
    b) MRI of all of the ducts
    c) Correlation of the milk duct orifices and ductal anatomy from subsequent studies with our previous work in lactating breasts and ductograms (IDEA grant).

2. Application of this work
   a) in a study of women with mammographic microcalcifications who are scheduled for biopsy. This protocol is pending approval from the UCLA IRB .
   b) by identifying and cannulating ducts in women scheduled for breast surgery (pending approval)

References

1. Imayama S, Mori M, Ueo H, Nanbara S, Adachi Y, Mimori K, Shimozono Y, Hori Y, Sugimachi K. Presence of elevated carcinoembryonic antigen on absorbent disks applied to nipple area of breast carcinoma patients. Cancer 78(6):1229-1234, 1996.

Appendix: Table 1
Fluorescence Experiments Data Sheet

1.) Sample: Porcine nipple(s)
1° antibody: hmw cytokeratin
2° antibody: FITC - IgG

Results: Experimental samples exhibited a fluorescent "hue" on the nipple surface.

2.) Sample: Porcine nipples(s)
a.) 5% acetic acid in saline 24hr pretreatment
b.) 5% acetic acid in saline 24hr pretreatment
c.) saline only
1° antibody: hmw cytokeratin
2° antibody: FITC - IgG

Results: Experimental samples a.) and b.) exhibited a fluorescent "hue" on the nipple surface. Sample c.) exhibited slight (pinpoint) fluorescence.

3.) Sample: Porcine nipple(s)
1° antibody: Epithelial membrane antigen (EMA)
2° antibody: FITC - IgG

Results: Inconclusive (inconclusive results were found to be due to the lack of cross reactivity of EMA with porcine)

4.) Sample: Porcine nipples(s)
a.) dekeratinization
b.) non dekeratinized
1° antibody: lmw cytokeratin
2° antibody: FITC - IgG

Results: Entire surface of experimental sample a.) fluoresced.

5.) Sample: Porcine nipples(s)
c.) dekeratinization
d.) non dekeratinized
1° antibody: lmw cytokeratin
2° antibody: FITC - IgG

Results: Entire surface of experimental sample a.) fluoresced and no fluorescence was seen on experimental sample b.).

Appendix: Table 2
Duct Identification

Experiment	Ducts Identified	Ducts Cannulated	Ducts Confirmed
1	5	-	-
2	6	-	-
3	3	2	2
4	2	-	-
5	3	2	2
6	3	-	-
7	4	3	3
8	5	1	1
9	7	7	7
10	6	6	6
11	6	6	6
12	8	8	8
13	7	7	7

Appendix: Table 3
Patient Data Summary

Note the 2000 series includes UCLA patients undergoing lumpectomy. The 3000 series is UCLA patients undergoing mastectomy and the 4000 series includes Olive View patients undergoing mastectomy or lumpectomy. As of this time we have had no known complications from this procedure.

Study number	date	side/op	Ducts cannulated	ducts washed	cytology	comments	wash	ductal	cells
UCLA									
3000	12/12/9	R mast	1	a	acellular		1	0	0
3001	1/6/9	R mast	3	a	RBC's,foam cells, atypical ductal cells	testing different collection devices	1	1	1
				b	foam cells, duct cells		1	1	1
		L mast	3	a	well cohesed ductal epithelial cells, ? DCIS		1	1	1
3002	1/16/9	L mast	2	a	scant scattered epithelial cells		1	1	1
				b	**ductal epithelial cells**		1	1	1
3003	1/21/9	R mast	3	a	foam cells, clusters of ductal cells		1	1	1
				b	acellular		1	0	0
3004	2/12/9	L mast	5	a	**ductal epithelial cells**		1	1	1
		R mast		b	foam cells, lymphocytes		1	0	1
3005	2/20/9	R mast	1	a	acellular		1	0	0
		L mast	3	a	acellular		1	0	0
3006	7/28/9	L mast	4	a	rare poorly perserved cells		1	0	1
				b	small clusters of ductal epithelial cells		1	1	1
3007	8/28/9	L mast	2	a	**ductal epithelial cells**		1	1	1
3008	3/19/9	R mast	1	a	cytology not done	fluid used for analysis for markers			
3009	3/25/9	R mast							
		L mast	1	0		no washings obtained			
3010	4/6/9	R mast							
		L mast	2	b	cytology not done	fluid used for analysis for markers			

3011	4/8/9	L mast				specimen not used			
3012	4/24/9	L mast	3	0					
3013	4/29/9	R mast				unable to cannulate			
		L mast	1	0		removed keratin plugs			
3014	5/14/9	R mast	1	1	clusters of benign ductal epithelial cells	Ljung	1	1	1
3015	5/19/9	L mast	2	1	clusters of benign ductal epithelial cells	Ljung	1	1	1
		R mast	1	1	benign ductal epithelial cells	Ljung	1	1	1
3016	6/24/9	mast	2	0					
3017	6/29/9	mast	3	0					
3018	7/21/9	mast	7	0					
Olive View									
4001	8/11/9	R mast	2	1	sheets and clusters of ductal epithelial cells		1	1	1
4002	10/22/9	L mast	1		deteriorated cells		1	0	1
4003	10/22/9	R lump	3	1	deteriorated cells		1	0	1
4004	10/22/9	L lump	2	1	deteriorated cells		1	0	1
4005	11/19/9	R lump	1	1	RBC's, acellular		1	0	1
4006	11/19/9	R mast	2	0					
		L mast	1	0					
4007	2/5/9	L lump	1	a	benign ductal epithelial cells	Ljung	1	1	1
4008	2/5/9	R lump	1	a	Benign ductal epithelial cells	Ljung	1	1	1
4009	2/18/9	R reex	2	a	**ductal cells**		1	1	1
4010	2/18/9	R lump	3	a	acellular		1	0	0
4011	2/18/9	L lump			macrophages, no duct cells	thick discharge no wash done			
		R							
4012	2/20/9	L mast	1	a	foam cells, aggregates of ductal cells		1	1	1
4013	2/20/9	R lump	2	a	foam cells, rare sheet of ductal epithelial cells		1	1	1
				NAF	foam cells				

4014	5/27/9	R lump	1	a		package destroyed in mail				
		L lump	1	a		package destroyed in mail				
4015	6/5/9	L mast	1	0						
4016	6/10/9	R lump	1	0						
4017	6/10/9	L lump	1	a	poorly perserved benign ductal epithelial cells		Ljung	1	1	1
4018	6/10/9	L lump	1	a	clusters of benign ductal epithelial cells		Ljung	1	1	1
4019	6/10/9		1	0						
4020	6/19/9		0	0						
4021	6/19/9		0	0						
							totals	31	20	26

Washings

pt #	date	diagnosis	# washed	unsuccessful	acellular	ductal	degenerate	other	total cells
2001	3/21/95	cancer	1		1				
2002	3/21/95	cancer	3		1	2			2
2003	3/21/95	cancer	1			1			1
2004	4/10/95	cancer	0						0
2005	4/10/95	cancer	1					1	1
2006	4/10/95	cancer	1	1					0
2007	4/24/95	phylloides	1		1				0
2008	5/8/95	dcis	0						0
2009	5/8/95	cancer	1	1					0
2010	5/16/95	cancer	1				1		1
2011	5/16/95	cancer	0						0
2012	5/16/95	cancer	0						0
2013	5/23/95	cancer	0						0
2014	5/30/95	cancer	1			1			1
2015	6/6/95	fibroadenoma	1			1			1

Washings/continued

Pt #	Date	Diagnosis	# Washed	Unsuccessful	Acellular	Ductal	Degenerate	Other		Total cells
2016	6/27/95	cancer	0							0
2017	6/19/95	cancer	1						1	1
2018	6/19/95	cancer	0							0
2019	6/19/95	cancer	0							0
2020			1							0
2021	6/26/95	cancer	1						1	1
2022	7/17/95	cancer	1	1						0
2023	12/12/95	cancer	1					1		1
2024	12/12/95	cancer	1	1						0
2025	1/18/96	cancer	3		2	1				1
2026	1/22/96	cancer	0							0
2027	1/23/96	cancer	1			1				1
2028	2/5/96	cancer	1	1						0
2029	2/6/96	dcis	1			1				1
2030	2/13/96	cancer	1	1						0
2031	2/5/96	dcis	0							0
2032	2/13/96	dcis	1		1					0
2033	3/5/96	cancer	0							0
2034	3/1/96	cancer	1			1				1
2035	3/26/96	cancer	0							0
totals			27	6	6	9	2	3		14
per cent				22%	22%	33%	7%	11%		52%

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 2 Feb 01

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCA, 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statements

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for the following grant numbers:

96-C-6117	ADB233743, ADB243030, ADB259960
94-J-4113	ADB251394, ADB241076, ADB220530

Request the limited distribution statement for Accession Document Numbers listed be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by email at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management